LEADING ARTICLE

Molecular testing for antibiotic resistance in *Helicobacter pylori*

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An estimated 7.5 million individuals in England and Wales are actively infected with *Helicobacter pylori* and hence knowledge of local resistance rates is of growing importance. Also, information on strain resistance following treatment failure is crucial in selecting an appropriate regimen as the development of bacterial resistance to antibiotics makes retreatment difficult. Molecular test methods may have an impact on improving the availability and accuracy of information on *H pylori* antimicrobial resistance to guide in the selection of primary as well as secondary backup treatment regimens.

Much publicity is currently given to the widespread use of antibiotics and the threats posed by the emergence of pathogenic organisms resistant to all available antibiotics. While it is generally accepted that treatment to eradicate *Helicobacter pylori* in patients with proved peptic ulcer is cost effective and benefits the patient and society, treatment to eradicate the organism in patients with non-ulcer dyspepsia (NUD) is a topic of ongoing debate.[1 2] One reason used in the argument against the test and treat strategy is that empirical or indiscriminate antibiotic treatment of NUD could have an undesirable outcome in the emergence and persistence of resistant strains of *H pylori* as well as the development of other resistant organisms in the gastrointestinal tract.[3] An estimated 7.5 million individuals in England and Wales are actively infected with *H pylori*[4] and so knowledge of local resistance rates is of growing importance to guide frontline treatments that include the macrolide clarithromycin, the 5-nitroimidazole metronidazole, as well as amoxicillin and tetracycline. Two of these antibiotics are used for *H pylori* primary eradication in combination with a proton pump inhibitor in triple therapy regimens.[5] Likewise, information on strain resistance following treatment failure is crucial in selecting an appropriate regimen as the development of bacterial resistance to antibiotics makes retreatment difficult.[5 6]

The clinical relevance of antibiotic resistance is also a controversial issue although its importance on outcome of therapy has been highlighted in several recent meta-analyses. For instance, van der Wouden and colleagues[7] addressed the influence of nitroimidazoles on the efficacy of treatment, based on world literature from 1993 to 1997, and calculated that eradication rates were 90% in metronidazole susceptible strains but <75% in resistant strains, although the choice of other drugs and treatment duration influenced the impact of resistance on treatment efficacy. A second meta-analysis based on studies published from 1983 to December 1997 to define the effect of pretreatment resistance to either metronidazole or clarithromycin on the success of therapy found that metronidazole resistance reduced effectiveness by an average of 37.7% but most striking was the finding that clarithromycin resistance reduced effectiveness by an average of 55%.[8] In a recent German study, 86% of 554 isolates from patients in whom one or more eradication therapies had failed were resistant to both clarithromycin and metronidazole.[9] This finding was consistent with data on isolates from biopsies submitted to our laboratory following failed primary treatment which indicated a dual (clarithromycin/metronidazole) resistance rate in the order of 72% for 22 patients in England over the past 18 months.

The purpose of this article is to consider the molecular test methods that might have an impact on improving the availability and accuracy of information on *H pylori* antimicrobial resistance to guide in the selection of primary as well as secondary backup treatment regimens.

WHY USE MOLECULAR METHODS OF SUSCEPTIBILITY TESTING?

H pylori is a relatively fastidious and slow growing microaerophilic microorganism and therefore standard culture based in vitro antibiotic susceptibility tests (disk diffusion, agar dilution, and Epsilometer-test methods), even in the hands of experts, are slow and can take at least 10–14 days from initial receipt of the gastric biopsy to reading and reporting the sensitivity results.

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The lack of a standard method for determination of susceptibility adds a further complication to understanding the relationship between clinical

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Abbreviations: NUD, non-ulcer dyspepsia; MIC, minimum inhibitory concentration; PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism; LiPA, line probe assay; DEIA, DNA enzyme immunoassay; LC, LightCycler.

outcome and in vitro susceptibility. Adoption of molecular based diagnostic assays for resistance detection therefore offers an attractive alternative approach to obtain susceptibilities to antibiotics with greater accuracy and speed, and the possibility of a same day result if tests are performed directly on the biopsy without culture. The success of such strategies has already been demonstrated for other particularly slow growing human pathogens causing a chronic infection such as *Mycobacterium tuberculosis*.[10]

MOLECULAR ASSAYS FOR CLARITHROMYCIN RESISTANCE

The introduction of molecular methods for *H pylori* antibiotic resistance testing has been facilitated by the fact that the mode of action of clarithromycin is well understood and is due to various point mutations in the peptidyltransferase region of domain V of the 23S rRNA gene. *H pylori* has two copies of that gene and the mechanism of resistance to clarithromycin appears to be decreased ribosome binding of the macrolide so that it fails to act by interrupting protein biosynthesis. In vitro determination of resistance to clarithromycin by conventional phenotypic tests generally gives reproducible results with a clear cut off point and so provides an accurate basis for validation of molecular assays. Isolates are considered to be resistant when the minimum inhibitory concentration (MIC) is >2 μg/ml, and inter-laboratory reproducibility appears to be good even though the precise MIC value used may vary between laboratories.

Molecular assays for detecting clarithromycin resistance in *H pylori* are all based on detection of mutations in the 23S rRNA genes. The basic assay, first described in 1996,[11] utilises a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) approach in which the region of the gene containing the mutations is amplified and then digested with restriction endonucleases that cut specifically at the mutation sites. The sizes of the resultant fragments indicate the presence/absence of a particular mutation—for example, the presence of the A2142G mutant creates an *Mbo*II site giving two comigrating fragments of about 700 bp whereas the presence of the A2143G mutant creates an additional *Bsa*I site giving major fragments of about 700 and 300 bp. This basic form of assay has been used successfully to test isolates of *H pylori* from patients in a number of different countries, and the association between resistance phenotype and the presence of specific mutations has been validated by direct sequencing.[11–14]

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Further interest in this molecular approach was stimulated by the finding from several groups of workers that the A2142G mutation was significantly more likely to be associated with higher levels of clarithromycin resistance[15 16] which might be attributable to different steric effects of the mutations at the binding sites. Our laboratory results for isolates of *H pylori* from England also indicate that the A2142G mutation is typically associated with higher level clarithromycin resistance (MICs >256).

A significant development was the discovery that the PCR-RFLP assay could be successfully applied to evaluation of clarithromycin resistance without culture by direct analysis of DNA extracted from gastric juice[17] and from gastric biopsies.[18–20] Although such an approach is significantly faster with same day results, a feasible possibility, there is a risk that PCR efficiency may be affected by the presence of inhibitors from the clinical specimens, and that specificity is reduced by the presence of high levels of non-*H pylori* DNA, notably mammalian host DNA.

Table 1 Molecular methods for detection of point mutations associated with clarithromycin resistance in *Helicobacter pylori*

PCR-restriction fragment length polymorphism (RFLP)
PCR oligonucleotide ligation assay (OLA)
DNA enzyme immunoassay (DEIA)
PCR line probe assay (LiPA)
PCR preferential homoduplex formation assay
Fluorescent in situ hybridisation
Real time PCR hybridisation assay using the LightCycler
DNA sequencing by conventional and real time (pyrosequencing) techniques

PCR, polymerase chain reaction.

DEVELOPMENT OF IMPROVED ASSAYS FOR CLARITHROMYCIN RESISTANCE

The basic PCR-RFLP assay for detection of the two commonest A to G mutations has undergone various modifications to improve speed and specificity. For instance, a 3' mismatched PCR using an additional specific primer was applied to detect the rarer A2142C mutation.[21] Alternative restriction enzymes have been used, notably *Bbs*I instead of *Mbo*II to improve detection of the A2142G mutation.[18 22] More important have been the developments of alternative formats to improve detection of specific mutations, such as the various hybridisation assays using oligo probes for extracted DNA and in situ hybridisation (table 1). Many of the assays are based on the principle of reverse hybridisation with labelled probes for up to seven mutations and the wild type, immobilised either in microtitre wells (DEIA) or on nitrocellulose (LiPA). In these assays, PCR products were hybridised to the probes at highly stringent conditions and the resultant hybrids detected colorimetrically. In the DNA enzyme immunoassay (DEIA), the detection system was an enzyme linked immunoabsorbent assay with a labelled antidouble stranded DNA monoclonal antibody. Biotinylated probes for the wild type and three mutations (A2142C, A2142G, and A2143G) were designed to test for mutations in DNA extracted from cultures of *H pylori*[22] and for use in a rapid (one day) laboratory assay that could be applied directly to gastric biopsies so avoiding the need for culture.[23]

The PCR line probe assay (LiPA) system is an alternative simple and cost effective way of detecting seven distinct resistance mutations and is highly suitable for testing large numbers of samples.[24] Other probes for targets related to pathogenicity such as *cag*A and *vac*A alleles can be added to the strips and so isolates can be simultaneously tested for several genotypic features. The probes were biotinylated and hybrids were detected by the streptavidin-alkaline phosphatase conjugate/substrate colorimetric system. The high degree of accuracy of the LiPA was recently demonstrated in a multinational study in six countries with positive and negative predictive values higher than 97% for detection of macrolide resistance.[25] The dominant mutations were A2143G (45%) and A2142G (33%) as well as a small number of A2142C mutations (2%). There was no significant difference between strains from different geographical locations (Europe, Australia, and Brazil). Interestingly, about 20% of strains contained multiple mutations which could be interpreted either as the presence of multiple strains or as different mutations in either copy of the 23S rDNA.

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The assay was recently applied to *H pylori* from patients in the west of Ireland showing a high clarithromycin resistance rate

of 27%, although conventional culture based methods were not performed in parallel.[26] Other more complex microtitre plate based systems such as the PCR oligonucleotide ligation assay using labelled capture and reporter probes,[16] and the preferential homoduplex formation assay using double labelled amplicons[27] have also been applied to mutation detection but have not been generally evaluated. However, the latter study demonstrated the advantages of testing DNA extracted from gastric juice as it may be more representative than a single gastric biopsy for detecting mixed (resistant/wild type) populations.

Another approach is the use of in situ hybridisation with fluorescently labelled 16S and 23r RNA targeted probes which has been proved to be a reliable and rapid means of in situ detection of *H pylori* and the main mutations associated with clarithromycin resistance.[28] Compared with PCR based approaches, this technique does not require extensive DNA preparation and allows detection in intact tissue sections within three hours. However, possible disadvantages of the technique are requirements for specific fluorescently labelled probes and an epifluoresence microscope.

A recent and conceptually unique approach to the rapid detection of mutations is provided by the LightCycler (LC) real time PCR hybridisation assay, which is a novel combination of real time PCR and hybrid thermal analysis performed in a single closed reaction capillary.[29] A 23S rDNA fragment is amplified in the presence of the fluorophore SYBR Green 1 and then hybridised to a probe labelled with a second fluorophore, Cy5. The temperature within the reaction capillary is then increased and the temperature at which the Cy5 fluorescent signal drops sharply indicates the point at which the probe dissociates (melting temperature). When there are mismatches present in the target sequence due to the presence of resistance associated mutations, the melting temperature is lower than that of a perfectly matched hybrid.

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One hundred UK isolates of *H pylori* were examined in an evaluation of the assay performed in the LC (Idaho Technology instrument), and mutations were detected in 33/34 (97%) of the isolates resistant to clarithromycin by conventional phenotypic assays.[29] The LC assay was simple to perform, and offered the advantage of detecting the rare A2143C mutation for which there is no restriction enzyme based assay as well as the two most common A to G mutations. After primary culture and DNA extraction, the LC assay could be completed in less than one hour, thereby overcoming the delays associated with conventional culture methods and so was significantly faster than the various other hybridisation assays. Recent studies confirm that the LC assay can also be applied successfully to direct analysis of *H pylori* DNA extracted from gastric biopsies without the need for culture.[30] Research is currently in progress to adapt these assays for use in the LC (Roche instrument) which utilises a dual probe/fluorophore detection system.

In contrast with all the above approaches, DNA sequencing provides the gold standard reference method for mutation detection although it is not technically feasible or cost effective for routine laboratory determination of *H pylori* resistance markers. Nevertheless, knowledge of nucleotide sequences has proved invaluable for validation of the various assays mentioned above, particularly for investigating where a resistant phenotype was not associated with any of the more common mutations.

A recent novel development in rapid sequencing based on the principle of pyrosequencing, a real time DNA sequence analysis of short (25–30 bp) DNA stretches, has been applied in rapid identification of *H pylori*[31] and its potential has been demonstrated for clarithromycin resistance typing of *H pylori* (http://www.pyrosequencing.com). Available data suggest this new technology can offer an accurate and rapid technique for sequence analysis of PCR amplicons providing easily interpreted results within hours.

MOLECULAR ASSAYS FOR DETECTION OF METRONIDAZOLE RESISTANCE

Primary resistance to metronidazole in *H pylori* is significantly high in many countries with reported rates of between 10% and 70%.[32] There are conflicting views on the clinical impact of metronidazole resistance on eradication; nevertheless, there is growing evidence that resistance seriously decreases the efficacy of metronidazole containing regimens.[8 33 34] Increased rates of resistance are attributed to repeated administration of metronidazole in the treatment of non-*Helicobacter* infections in regimens that are only partly inhibitory, leading to selection for resistance to *H pylori.*

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In England for instance, a 12 month survey of *H pylori* from two London centres indicated that rates were 59% for metronidazole compared with 12% for clarithromycin.[35] The London survey covered an ethnically heterogeneous population and therefore rates were significantly higher than those for mid Essex of 37.1% and 4.2%, respectively, with no significant upward trends over a five year period.[36] Similar rates have been reported in mainland Europe and the USA, although incidence can be far higher in developing countries and in certain immigrant populations.[31]

The most important step in the antimicrobial action of 5-nitroimidazoles such as metronidazole is the reductive activation of the nitro group, converting it from an inactive prodrug to a cytotoxic nitroso free radical form, with donation of electrons determined by the redox system of the target cell.[37] It has been suggested that resistance in *H pylori* may result from different alterations in the *rdxA* gene, which encodes an oxygen insensitive NADPH nitroreductase.[38 39] These alterations, which include missense and frameshift mutations, deletions, and insertions of transposable elements, generate a premature stop in the translated protein thereby resulting in a truncated and inactivated form of RdxA.[40–42] However, investigation of *rdxA* sequences from 30 independent isolates in Switzerland showed that no particular nucleotide mutation or amino acid substitution could be associated with metronidazole resistance.[43] It is also possible that other genes or mechanisms may be involved in the generation of resistance although there is no evidence of any direct role by auxiliary resistance genes. While inactivation of reductase encoding genes, notably *fdxB* (encoding ferrodoxin-like protein) and *frxA* (encoding NADPH flavin nitroreductase) can enhance high level resistance, genes that confer metronidazole resistance without *rdxA* inactivation are apparently rare or non-existent in *H pylori,* even when tested for in diverse populations.[40]

Because the molecular mechanisms of metronidazole resistance in *H pylori* are not yet fully understood, and certainly cannot be attributed to simple predictable genetic changes, it is currently not possible to design straightforward and rapid laboratory assays for detection of metronidazole resistance.

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However, there is new evidence to indicate that a system could be developed based on detection of the RdxA protein by immunoblotting with specific anti-RdxA antibody.[44] A 24 kDa immunoreactive band corresponding to the RdxA protein was observed in all metronidazole sensitive strains but was absent in most resistant isolates. Testing for the absence of RdxA protein, possibly in the form of a biopsy based assay, therefore would provide a novel molecular approach in identifying clinical isolates that would respond poorly to metronidazole containing regimens.

Estimation of in vitro metronidazole susceptibility therefore remains dependent on conventional culture based methods which continue to pose a number of reproducibility and interpretation problems, as evident from the lack of general agreement between results performed on the same strains of *H pylori* in different laboratories.[45] Uncertainty arises mainly from lack of standardised protocols for transportation of biopsies, for culture, and for performance of tests. For instance, some laboratories preincubate anaerobically which can cause false sensitivities. Another source of discrepancies is lack of agreement on cut off MIC levels for in vitro metronidazole susceptibility tests, and the problem of interpretation of intermediate sensitivities that may be due to the presence of mixed sensitive/resistant cultures. Recently, recommendations have been made to improve standardisation of in vitro metronidazole susceptibility testing.[46]

TESTS FOR OTHER ANTIMICROBIALS

Resistance in vitro to tetracycline or to amoxycillin appears to be rare in clinical isolates of *H pylori*. Nevertheless, naturally occurring amoxycillin resistant *H pylori* have been reported from different geographical locations but while most resistances were not stable on storage, there is evidence of the emergence of stable resistance to amoxicillin in the USA[47] as well as the emergence of high level amoxicillin resistance (MICs >265 μg/ml) in Germany.[48]

> "Resistance in vitro to tetracycline or to amoxycillin appears to be rare in clinical isolates of *H pylori*"

Amoxicillin is an extensively used antibiotic and development of resistance by *H pylori* could have a major detrimental effect on treatment success. Some regimens have been developed that replace proton pump inhibitors with bismuth compounds.[4] The antimicrobial action of bismuth compounds is related to expression of metallic transporter proteins in the cytoplasmic membrane, and to disruption of the glycocalyx-cell wall.[31] However, it is not known if *H pylori* strain specific differences might contribute to treatment failure, and at present there are no molecular tests for determining susceptibilities to any of these antimicrobials.

CONCLUSIONS AND THOUGHTS FOR THE FUTURE

After two decades of experience in the eradication of *H pylori* infections, there is still a paucity of systematic information on primary antibiotic resistance in the UK and most other countries. A positive development has been the growing awareness that resistance to clarithromycin and metronidazole (individually or in combination) can significantly reduce the efficacy of common eradication regimens for *H pylori* and that resistance to clarithromycin, in particular, is an important predictor of treatment failure.

> "The indications are that new molecular tests using real time PCR and sequencing will have an expanding role in the rapid detection of resistance to clarithromycin"

This fact was highlighted by the recommendation of the Maastricht 2-2000 Consensus Report (under public health issues) that resistance programmes should be implemented since clarithromycin resistance affects the efficacy of firstline therapy, with the conclusion that high prevalence of resistance may require the performance of testing before initiating treatment. The indications are that new molecular tests using real time PCR and sequencing will have an expanding role in the rapid detection of resistance to clarithromycin, particularly if results can be obtained directly from gastric biopsies without the need for culture, and they can identify the risk of high level resistance as well as providing evidence of mixed infections. Such information will be invaluable to guide individual patient treatment and to gain a better understanding of the effect of resistance on outcome. The availability of reliable molecular tests for detecting metronidazole resistance are awaited as a panel of assays for both antibiotics would strengthen the case for more routine use of molecular tests, particularly in combination with automated DNA extraction. The benefits of a molecular test for markers of amoxicillin resistance also need to be explored. Likewise, improved systematic surveillance of resistance both nationally and internationally is needed to monitor trends with time and to identify high risk population groups. Such information is especially important to guide empirical treatment in local practice as there are no new agents of note under development. At present, collection of data is limited by the availability of gastric biopsies and therefore susceptibility information is generally fragmented and unrepresentative. Structured prospective studies are essential to improve the quality of data based on both resistance phenotype and associated molecular markers. Moreover, the use of non-culture based molecular methods to test alternative clinical samples, such as stools or gastric juice, needs to be explored as a means of monitoring resistance markers. The spectre of treatment options becoming exhausted has been raised[49]—rapid and specific molecular testing for antibiotic resistance may therefore be an important factor in ensuring this eventuality is avoided.

Note: for reasons of consistency and to facilitate comparisons between different studies, the sequence notations for defining positions of mutations in the 23S rRNA gene follow those proposed by Taylor and colleagues[50] from the *H pylori* sequence rather than those based on the *Escherichia coli* sequence.

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